Single-cell attachment and culture method using a photochemical reaction in a closed microfluidic system

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Recently, interest in single cell analysis has increased because of its potential for improving our understanding of cellular processes. Single cell operation and attachment is indispensable to realize this task. In this paper, we employed a simple and direct method for single-cell attachment and culture in a closed microchannel. The microchannel surface was modified by applying a nonbiofouling polymer, 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, and a nitrobenzyl photocleavable linker. Using ultraviolet (UV) light irradiation, the MPC polymer was selectively removed by a photochemical reaction that adjusted the cell adherence inside the microchannel. To obtain the desired single endothelial cell patterning in the microchannel, cell-adhesive regions were controlled by use of round photomasks with diameters of 10, 20, 30, or 50 μm. Single-cell adherence patterns were formed after 12 h of incubation, only when 20 and 30 μm photomasks were used, and the proportions of adherent and nonadherent cells among the entire UV-illuminated areas were $21.3\% \pm 0.3\%$ and $7.9\% \pm 0.3\%$, respectively. The frequency of single-cell adherence in the case of the 20 μm photomask was 2.7 times greater than that in the case of the 30 μm photomask. We found that the 20 μm photomask was optimal for the formation of single-cell adherence patterns in the microchannel. This technique can be a powerful tool for analyzing environmental factors like cell-surface and cell-extracellular matrix contact. © *2010 American Institute of Physics.* [doi:10.1063/1.3494287]

I. INTRODUCTION

Cells are the basic units of all living organisms and are essential to all biological processes. Many cellular behaviors are modulated by environmental conditions; understanding these relationships will provide an approach to predict the fundamental properties of cells. However, most conventional cell-based biological analyses are averaged across large groups of cells, even though each single cell exists in its own microenvironment, such that population studies cannot adequately represent the conditions of the individual cell. This averaging of various cell characteristics in bulk conditions can be misleading, when the precise conditions of the single cell are overlooked. For this reason, single-cell analysis has become the focus of advanced cell biological

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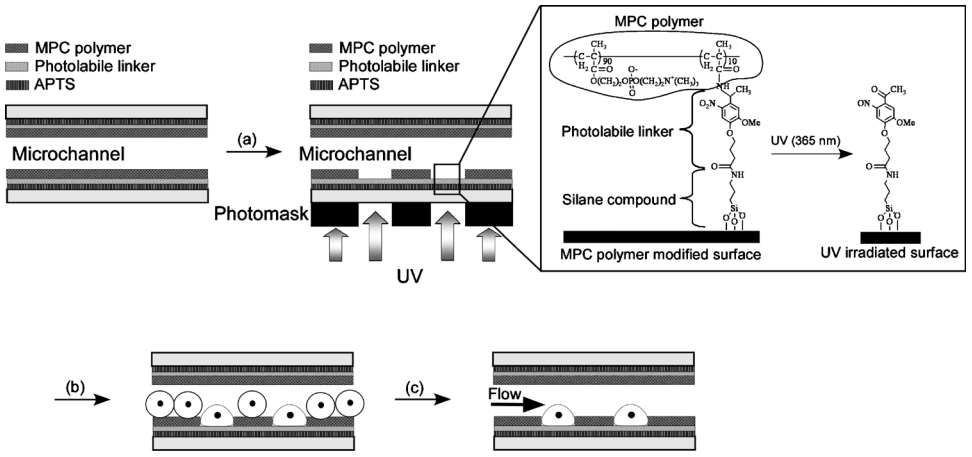

FIG. 1. Schematic illustration of single-cell patterning in a microchannel. (a) UV was illuminated through a photomask. (b) Introducing EC suspension inside the microchannel and incubation for 2 h. (c) The fresh medium was connected to the microsyringe pump at a flow rate of 0.2 μl/min for 12 h.

research in recent decades. Microfluidic systems have become popular because of their ability to facilitate detailed understanding of biological processes and the mechanisms underlying cellular activities. Microfluidic systems can be used to demonstrate the feasibility of integration of the multiple steps involved in single-cell analysis,[1–10] such as separating, positioning, stimulating, and detecting, which are impossible with other widely used single-cell analysis methods such as automated microscopy,[11] flow cytometry,[12] and laser scanning cytometry.[13]

Numerous microfluidic-based single-cell analysis methods have been developed and can be classified according to the positioning methods, such as electrical positioning,[14] hydrodynamic trapping,[15] physical trapping,[16,17] and microwell trapping.[18,19] However, these methods are inappropriate for the detection of environmental factors such as cell-cell contact and cell-extracellular matrix (ECM) contact. Cell contact on the surface plays a pivotal role in the behavior of adherent cells, as adhesion triggers intracellular signal transduction, affecting cellular growth, differentiation, and proliferation. In order to investigate these conditions at the single-cell level, positioning and surface attachment of single cells are required. Moreover, artificial structures for trapping are not suitable for observations of cell communication between individual cells under external stimuli conditions.

Single-cell attachment and culture techniques have also been reported previously;[20–22] however, these methods are typically employed not in microchannels, but on flat substrates. To achieve sensitive and highly efficient single-cell analysis at the point of cell contact, microfluidic systems, single-cell patterning, and cell culture techniques must be combined. In order to establish the desired pattern, it is necessary to use external stimuli to induce cell micropatterning inside the sealed microchannels. This enables *in situ* surface micropatterning directly to the microchannel without having to attach and detach the plate. Using UV exposure, nonbiofouling compounds can be applied in selective patterns to regulate cell adhesion inside the microchannel. Furthermore, this approach would allow the cells to communicate freely in response to external stimuli without being isolated in wells or physical structures.

In this paper, we present a new single-cell patterning method in a microchannel. We have previously reported effective surface modification methods to achieve cell micropatterning directly inside the microchannel via photochemical reaction.[23] This technique is based on the combination of a nonbiofouling polymer, 2-methacryloyloxyethyl phosphorylcholine (MPC),[24,25] and a nitrobenzyl photocleavable linker (PL).[26,27] The MPC polymer, PL, and silanization reagents are covalently bonded to the glass surface; the MPC polymer is selectively removed by UV irradiation, forming a cell-adherent hydrophobic surface.[28] This method can be used for the direct micropatterning of single cells in a microchannel (Fig. 1).

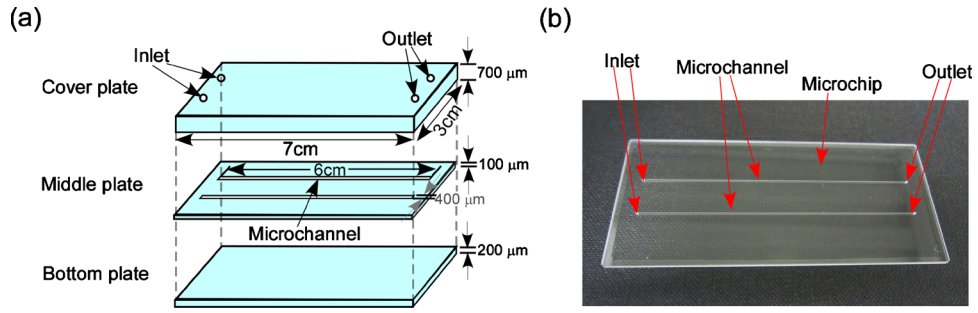

FIG. 2. (a) Components of a microchip. The thicknesses of the cover, middle, and bottom plates were 700, 100, and 200 μm, respectively. Inlet and outlet ports were mechanically etched on the cover plate. The middle plate was penetrated through its entire thickness with a linear channel 6 cm long, 400 μm wide, and 100 μm deep. The cover, middle, and bottom plates were thermally bonded to form a microchip. (b) Photographs of the fabricated microchip.

II. MATERIALS AND METHODS

A. Chip fabrication

The microchip was fabricated by mechanical etching. Briefly, the microchip was composed of three quartz glass plates (30 mm × 70 mm), i.e., the cover, middle, and bottom plates, with thicknesses of 700, 100, and 200 μm, respectively [Fig. 2(a)]. Two small access holes (diameter=600 μm) for the inlet and outlet of reagents as well as for cell introduction were mechanically bored into the cover glass plate. Using an endmill (ultraprecision mounted point) (FSK, Inc., Aichi, Japan), the 700-μm-thick middle plate was penetrated, and the penetrated microchannel was a linear shape, about 6 cm long, with a width of 400 μm, following the grinding of approximately 100 μm of thickness. After intensive washing and ultrasonication with de-ionized water, the middle plate was placed between the cover plate and the nonpatterned bottom plate, and was bonded using an optical contact; that is, the plates were polished to an optical smoothness and then laminated together with no adhesive in an oven at 1080 °C. The microchannel was linear in shape, about 6 cm long, with a planar cross section 100 μm deep and 400 μm wide [Fig. 2(b)].

B. Preparation of endothelial cells

Endothelial cells (ECs) were cultured in 60 mm cell culture dishes at 37 °C in 5% CO_2 in an EC culture medium (Lonza, Walkersville, MD). After the ECs reached confluence, they were washed with 2 ml HEPES (KURABO, Osaka, Japan) and detached from the cell culture dish by using 1 ml trypsin (KURABO). Detached cells were added to 4 ml trypsin inhibitor (KURABO), and the cell suspension was centrifuged at 1100 rpm for 5 min. The supernatant was discarded, followed by resuspension of the ECs in fresh medium at the required concentration for the subsequent experiments.

C. Surface modification inside the microchannel

The surface modification method was based on our previous work.[23] Experimental procedures were as follows. The microchip was assembled using a chip-holder [Fig. 3(a)] and was connected with capillaries. The microchannel was cleaned using a 0.1 M NaOH aqueous solution at RT for 30 min with a flow rate of 20 μl/min followed by rinsing with de-ionized water at 20 μl/min for 30 min. The inner surface of the microchannel was cleaned and then treated with 3% (v/v) 3-aminopropyl-triethoxysilane (Sigma-Aldrich Co., St. Louis, MO) in chloroform using a syringe at RT for 2 h, followed by washing with chloroform, ethanol, de-ionized water, and *N*,*N*-dimethylformamide (DMF) by using a microsyringe pump at 20 μl/min for 10 min each. To this amino-terminated surface, a nitrobenzyl photolabile linker was modified using 5 mM Fmoc-photolabile linker [4-(4-(1-(9-fluorenylmethoxycarbonylamino)ethyl-2-methoxy-5-nitrophenoxy)-

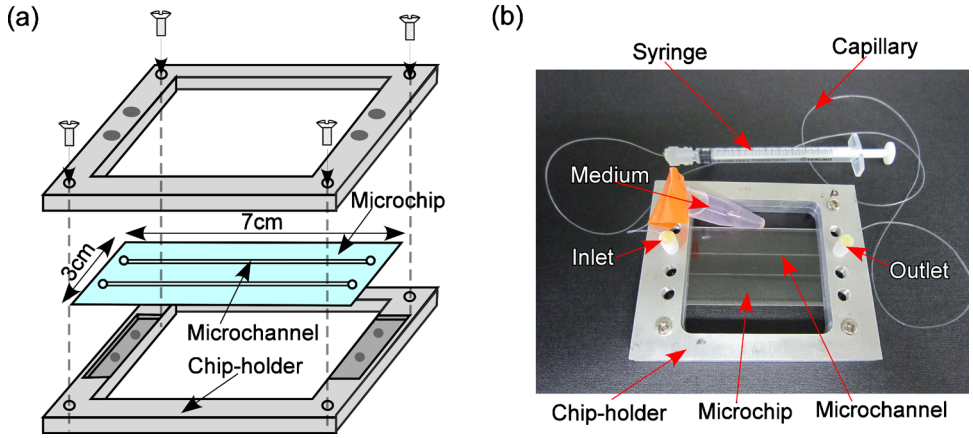

FIG. 3. Microfluidic system for cell culture. (a) Illustration of the microchip assembly with chip-holder. (b) Photographs of the microfluidic cell culture system. The microchannel and capillary were filled with medium.

butanoic acid)] (Advanced ChemTech, Louisville, KY), 10 mM benzotriazol-l-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (TCI, Tokyo, Japan), and 1-hydroxy-benzotriazole (TCI), *N*,*N*-diisopropylethylamine in DMF, applied for 3 h at RT and allowed to react for 2 h, followed by rinsing with DMF and methylene chloride at 20 μl/min for 10 min each.

Next, poly [2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-methacrylic acid] (Mw = 100 K, MPC: 90 mol %, methacrylic acid: 10 mol %, synthesized by the conventional radical polymerization technique) was grafted by 1-[3[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (TCI) coupling for 12 h, which created a nonfouling surface, which was then rinsed with de-ionized water at 20 μl/min for 60 min. After filling the microchannel with fresh medium [Fig. 3(b)], the MPC polymer-modified surface was exposed to UV light (365 nm, 1200 mW/cm^2); the light passed through a microchip plate with a photomask (chrome patterns on quartz), which was manufactured by the Institute of Microchemical Technology Co., Ltd. (Kanagawa, Japan). The microchip was reassembled using a chip-holder and was connected with capillaries and washed under flowing conditions (20 μl/min) using a combination of de-ionized water, then ethanol (for sterilization), then de-ionized water again for 30 min each. Before introduction of the cell suspension, all the capillaries and chip-holders were replaced with newly autoclaved ones to guard against contamination. The fresh medium was pumped into the microchannel, then the microfluidic system was incubated at 37 °C in 5% CO_2 for 1 h.

D. Cell micropatterning in a microchannel

Microfluidic chips were prepared as above. For EC patterning, round photomasks of varying diameters (10, 20, 30, or 50 μm) with black 100 μm separations were applied, and the cell concentration set at 6×10^5 cells/ml. Following UV irradiation, the cell suspension was introduced, and the capillary was clipped to inhibit accidental flow within the microchannel. Then, the microchannel was incubated at 37 °C in 5% CO_2 for 2 h, then connected to a microsyringe pump to introduce medium at 0.2 μl/min for 12 h.

E. Staining of cell nuclei

After 12 h of incubation, 4% paraformaldehyde in phosphate-buffered saline (PBS) was applied at 0.6 μl/min at RT for 30 min to fix the cells in the microchannel. Then, the microchannel was washed with PBS at 0.6 μl/min for 20 min. The cells were permeabilized by 0.5% Triton X-100 in PBS at 0.6 μl/min for 30 min and rinsed with PBS at 0.6 μl/min for 20 min. Nuclear staining was done with 2 μg/ml 4′,6–diamidino-2–phenylindole (DAPI) at 0.3 μl/min for 1 h, followed by washing with PBS at 0.6 μl/min for 20 min. For nuclei observations, the wave-

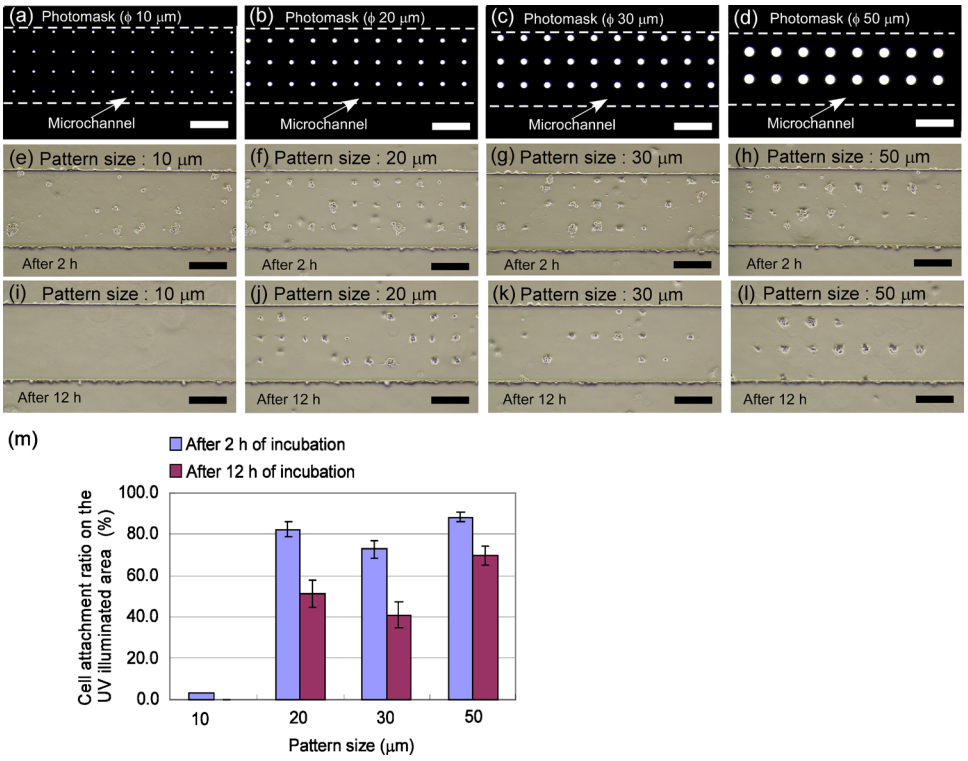

FIG. 4. Phase contrast images of micropatterned adhered ECs in the microchannel. Round photomasks with diameters of (a) 10 μm, (b) 20 μm, (c) 30 μm, and (d) 50 μm were used. After 2 h of static incubation [(e)–(h)], after 12 h of incubation at 0.2 μl/min flow [(i)–(l)]. (m) The rate of cell attachment on UV-illuminated areas according to pattern size. The error bars represent standard deviations. Scale bar=200 μm

lengths of both excitation (λ_{ex}) and emission (λ_{em}) spectra were λ_{ex}=350 nm and λ_{em}=465 nm (using a U-MNUA2 fluorescence mirror unit) for DAPI. The images were recorded through a complementary metal oxide semiconductor color camera (IK-HR1D, Toshiba). The images were saved using MVFLEXAVCIO software in MICROSOFT WINDOWS XP.

III. RESULTS

A. Single-cell patterning in a microchannel

Here, we tested direct single-cell patterning inside a closed microchannel. To ensure single-cell attachment, the cell-adhesive area was adjusted. UV light stimulated the removal of the MPC polymer from the microchannel surface. For cell immobilization, round photomasks of various diameters (10, 20, 30, or 50 μm) were used [Figs. 4(a)–4(d)]. The microchannel surface was UV-illuminated through the photomask, and the EC suspension was introduced. After 2 h of incubation, EC attachment initiated on the UV-illuminated area [Figs. 4(e)–4(h)]. At this point, the ratio of the attached cells on the UV irradiated areas was calculated. The results were 17.8% ± 3.1%, 82.6% ± 3.7%, 72.8% ± 4.2%, and 88.4% ± 2.4% for the pattern sizes of 10 μm, 20 μm, 30 μm, and 50 μm, respectively [Fig. 4(m)]. At least 70% of the UV-exposed patterns were occupied by cells, except in the case of the 10 μm photomask. The microchannel was connected to a microsyringe pump at a flow rate of 0.2 μl/min to provide a continuous flow of fresh medium to the ECs.

After 12 h of culture under flow conditions, the ECs remained attached to the UV-illuminated adherence patches [Figs. 4(j)–4(l)]; however, the ratio of attached cells on the UV-irradiated areas was reduced to 0.0%, 51.4% ± 6.7%, 41.0% ± 6.1%, and 69.6% ± 4.6% for the 10, 20, 30, and 50 μm photomasks, respectively [Fig. 4(m)]. All the cells attached on the 10 μm patterns were

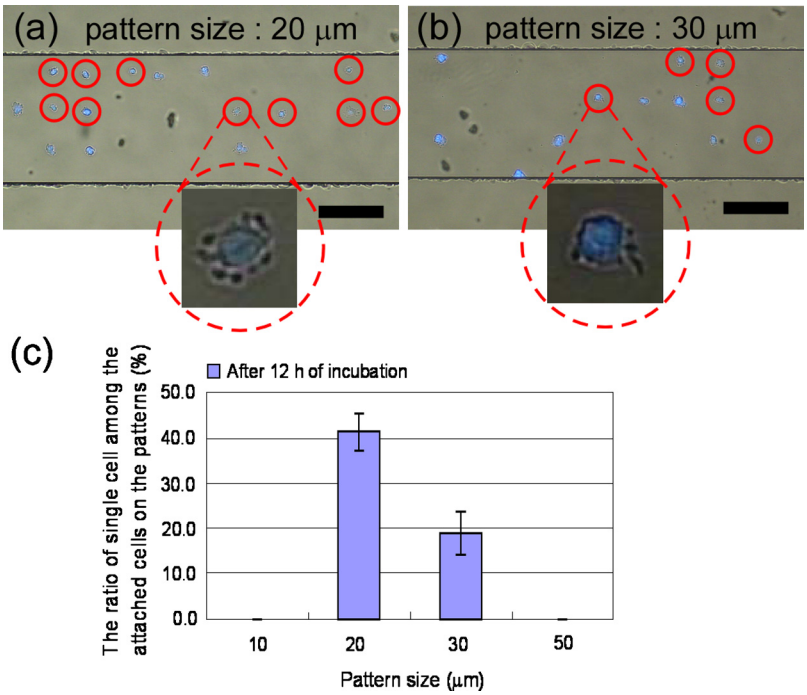

FIG. 5. Nuclei of the micropatterned ECs were stained with DAPI [(a) and (b)] after 12 h incubation at 0.2 μl/min. Round photomasks with diameters of (a) 20 μm and (b) 30 μm were used. Red circles indicate the single cells attached inside the microchannel. (c) The rate of single attached cells on the UV-illuminated areas. Scale bar=200 μm. The error bars represent standard deviations.

washed away under flow conditions. We previously reported the detachment of adhered cells after 12 h culture,[23,29] but succeeded in culturing ECs and in single-cell adherence patterns inside a microchannel at a flow rate of 0.2 μl/min. The strength of the cellular adherence to the surface was not strong enough to maintain the adherence under flow. We calculated the shear stress to be 0.02 dyn/cm^2 at this flow rate.[30] Although some portions of the cells were washed away under flow conditions, adherence was limited to the UV-illuminated areas, and no cells adhered to the MPC polymer-modified surface. The sizes of the adhered cell patches nearly coincided with the area exposed by the photomask.

B. Ratio of single-cell patterns in a microchannel

It was difficult to discriminate the single adhered cells, so the adhered cell nuclei were stained with DAPI at a flow rate of 0.3 μl/min for 1 h, followed by rinsing with PBS [Fig. 4(i)]. The stained cells were observed under a fluorescence microscope. When 20 and 30 μm photomasks were used, single adhered cells were observed [Figs. 5(a) and 5(b)]. Red circles indicate the single cells adhered inside the microchannel. The ratios of single cells for the 10, 20, 30, and 50 μm photomask patterns were 0.0%, 41.4% $\pm$ 4.1%, 18.9% $\pm$ 4.8%, and 0.0%, respectively [Fig. 5(c)]. Single cells were only observed with the 20 and 30 μm photomasks. If these results were recalculated to encompass the entire UV-illuminated area, the ratio of single-cell adherence was 21.3% $\pm$ 0.3% and 7.9% $\pm$ 0.3%, respectively. The 20 μm photomask yielded 2.7-fold greater adherence than the 30 μm photomask. We thus concluded that the 20 μm photomask was optimal to obtain single-cell adherence in the microchannel.

IV. DISCUSSION

To our knowledge, few reports of single-cell attachment and culture in microfluidic chip systems have been published.[31] These methods are generally performed by positioning the cells on

the substrate first, after which a polydimethylsiloxane cover plate, containing the microchannel, is applied. This process is not convenient and, as the microchannel is made smaller, it can become impossible to precisely place the adherent cells. In our technique, single-cell adherence was directly controlled inside the closed microchannel by using photochemical activation, and the technique has been demonstrated to work in a microfluidic system. By using this approach, the spatial distribution, shape, and number of adhered cells, from single cells to thousands of cells, can be regulated by controlling the cell adhesive regions inside the microchannel.

We attempted to improve the rate of single-cell adherence by increasing the concentration of the EC suspension to $12\times10^5-3\times10^6$ cells/ml before introduction. The attachment rate on UV-exposed areas was increased about 90%; however, the overall percentage of single adhered cells was 5%–9% on the entire UV-illuminated area, even with the 20 μm photomask (data not shown). More concentrated cell suspensions resulted in the attachment of multiple cells within each adhesive patch. When a relatively low number of cells were introduced (below 1×10^5 cells/ml), the rate of single-cell adherence was 9%–16% (with the 20 μm photomask, data not shown). We also found that the optimal cell density for ECs was $1\times10^5-3\times10^5$ cells/ml. These results indicated that the rate of single-cell adherence was limited to a specific range of cell suspension concentrations.

To apply this technique to sensitive and highly efficient single-cell analysis, culturing systems should be equipped with high-throughput capabilities and fully automated operation for the integration of multistep procedures such as cell seeding, cell culture, chemical stimulation, and detection. For example, the following functions could be added: (1) parallel microchannels with chambers for single cells (size of the chamber should be smaller than 50 μm) and (2) a concentration-response mode to create a concentration gradient in the channel.[32] Furthermore, this technique can be applied to real-time dynamic monitoring of stimulated release from the adhered single cells by combining micro- and extended nanochannels in a single chip.[33] The amount of secretion from a single cell is extremely small, and should be delivered to the extended nanochannel, which we have defined as 10–1000 nm. Short diffusion distances and the very large surface-to-volume ratio in this model could be beneficial for analysis. In the near future, such a modified system should serve as a valuable tool for single-cell operation and techniques for fundamental single-cell contact-based studies that analyze environmental factors and secretion release at the single-cell level.

V. CONCLUSION

A simple and direct single-cell patterning method has been developed for use in a microchannel. A surface modification method involving a MPC polymer and a photolabile linker was adopted for controlling cell adherence. The MPC polymer was effectively eliminated by UV illumination, and ECs selectively attached as single cells when round 20 or 30 μm photomasks were applied. Furthermore, we demonstrated that the 20 μm photomask was optimal for the formation of single-cell adherence patterns in the microchannel. This method can be applied to develop techniques for fundamental cell biology studies of the environmental conditions of individual cells such as cell-cell contact and cell-ECM contact.

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